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PRINCIPAL INVESTIGATOR: Albert B. DeLeo, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, Pennsylvania 15260

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6. AUTHOR(S)
Albert B. DeLeo, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pittsburgh
Pittsburgh, Pennsylvania 15260

E-Mail: deleo@imap.pitt.edu

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13. ABSTRACT *(Maximum 200 Words)*

Approximately 30% of breast cancer patients are p53 sero-positive and have detectable anti-p53 T cell proliferative responses. Tumors expressing mutant p53 molecules have an enhanced potential to present wild-type-sequence (wt) p53 epitopes derived from mutant p53 for T-cell recognition. Vaccines targeting these epitopes would be broadly applicable. HLA-A2.1-restricted CTL-recognizing wt $p53_{264-272}$ and $_{149-157}$ peptides have been generated from PBMC obtained from healthy donors and/or oral cancer patients. A subset of these donors were found to be non-responsive to the $p53_{264-272}$ peptide, and altered peptide ligands of this epitope were identified that induced CTL from PBMC that were non-responsive to the parental peptide. Currently, precursor CTL (pCTL) for the $p53_{264-272}$ epitope present in unstimulated PBMC can be identified by 4-color flow cytometry using soluble PE-conjugated HLA-A0201/p53 peptide tetrameric complexes (tetramers). An analysis of anti-p53 pCTL in the peripheral circulation and tumors of breast cancer patients was done with tetramers for the wt p53 $_{264-272}$ and $_{149-157}$ peptides. An analysis of genomic p53 exons 5-8 of the patients' tumors, when available, was also performed. The results of this study provide a basis for further investigation of the anti-p53 responses of breast cancer patients and will facilitate p53-based immunotherapy of breast cancer.

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Introduction

The focus of this research initiative was to assess the p53-directed anti-tumor CTL-mediated responses of breast cancer patients. The consensus that half of all human tumors contain alterations in p53 makes this tumor suppressor gene product an attractive candidate for development of a broadly-applicable cancer vaccine (1-3). Whereas p53 missense mutations may generate or create tumor-specific epitopes, the constraints of antigen presentation limits their vaccine applicability to individuals expressing the class I MHC restriction molecules capable of presenting these epitopes. In contrast, many tumors, including breast carcinoma cell lines, have the potential of enhanced presentation of non-mutated, wild type sequence (wt) p53 epitopes derived from the altered p53 molecules accumulating in the tumor. The detection of IgG-mediated anti-p53 antibody responses in ~20-30% of breast cancer patients is indicative, at minimum, of the occurrence in these patients of a $CD4^{+}$ T-cell-mediated anti-p53 immune response (4). This conclusion has been confirmed by the detection of anti-p53 proliferative T cell responses using PBMC obtained from p53 sero-positive patients . Whether anti-p53 CD8 T cells were involved in these responses is not known. This analysis of anti-p53 cellular immune responses of breast cancer patients was undertaken to provide insights into the potential of p53-based vaccines for immunotherapy of breast cancer.

Body

Flow cytometry analysis of lymphoid populations using soluble fluorochrome-conjugated class I MHC/peptide tetramers are facilitating the identification of anti-tumor peptide T-cells in unstimulated PBMC and *in vitro* stimulated (IVS) cultures of lymphocytes obtained from normal donors and cancer patients (5). In particular, this technology is developing into a useful diagnostic tool for monitoring T-cell mediated anti-tumor responses in cancer patients undergoing immunotherapy in the absence of extensive *in vitro* analyses of T-cell functions.

Five HLA-A2.1-restricted, CTL-defined wt p53 epitopes have been identified. These are p53 $_{65\text{-}73,\ 149\text{-}157,\ 217\text{-}225,\ 264\text{-}272\ \text{and}\ 322\text{-}330}$. The wt p53 $^{264\text{-}272}$ epitope has been the most extensively studied. Anti-tumor CTL recognizing the HLA-A2.1-restricted wt p53 $_{264\text{-}272}$ have been generated from PBMC of healthy donors (6-10) and, more recently in this laboratory, from PBMC obtained from HLA-A2+ oral squamous cell carcinoma (OSCC) patients, as well (11). Autologous DC pulsed with peptide are used in these studies for *in vitro* stimulation (IVS) of anti-p53 CTL. Our results indicate that anti-p53$_{264\text{-}272}$ CTL could be generated from 2/7 healthy donors and 3/7 patients tested. The reactivity of these CTL for the epitope, either pulsed onto HLA-A2+ target cells or naturally presented by tumors, was detected in standard ^{51}Cr-release cytotoxic assays, as well as ELISPOT for IFNγ assay. An interesting aspect of this analysis was the correlation between the PBMC responsiveness of patients and the p53 status of their tumors. Patients responsive to this epitope were found to have had tumors which had a low potential to present this epitope: namely, these tumors expressed normal levels of wt p53 or accumulated p53 expressing a missense mutation at codon 273 that has been shown to block processing of the epitope (12).

Our analysis has expanded to include identification of anti-wt p53$_{264\text{-}272}$ precursor CTL present in the peripheral circulation of donors using phycoerythin (PE)-labeled HLA-A2.1/ wt p53$_{264\text{-}272}$ tetramer complexes obtained from the NIH Facility. A four-color flow cytometry protocol, which was designed for detection of rare events, was used to analyze unstimulated PBMC obtained from healthy donors and OSCC patients (n=30 each). HLA-A2.1- flu matrix and -HIV peptide tetramers, also obtained from the NIH facility, were used in the analysis as positive and negative controls, respectively. Previously, it had been established using the flu and HIV peptide tetramers that specific tetramer binding to CD3+CD8+ T cells was associated with inhibition of the binding of the anti-CD3 mAb to the T cells (13). As a result, HLA-A2+ CD8+ tetramer+ cells had a lower level of CD3 staining than detected on CD8+ tetramer$^-$ T cells.

Based on the frequency of anti- wt p53$_{264\text{-}272}$ pCTL detected using the tetramer in the PBMC of HLA-A2.1$^-$ donor samples, a frequency of >1/8,000 was determined to indicate a positive level of tetramer+ cells in PBMC of HLA-A2$^+$ individuals. The average frequency of pCTL recognizing this epitope was found to be ~1:5000 in normal

donors, whereas the average frequency of oral cancer patients was determined to be ~1:3500. Although there was a marked difference in the frequencies between patients and normal donors, this difference was not significant. All of the patients whose PBMC t responded to in vitro induction of anti-$p53_{264\text{-}272}$ CTL had frequencies >1:3500. The p53 status of the patients' tumors was then determined and correlated with frequency of anti-p53 pCTL in their peripheral circulation. The results divided patients into two groups. One group of patients (6 of 23) had tumors with low potential to present the epitope and pCTL frequencies of ~1:1600, while the other patient group had tumors that were most likely able to present the epitope (accumulated mutant p53) and had an average anti-$p53_{264\text{-}272}$ pCTL frequency in their circulation of ~1:5000, a level comparable to that detected in normal donors[1]. Several possible explanations can account for the observed inverse potential between the responsiveness of an OSCC patient to the wt $p53_{264\text{-}272}$ epitope and the ability of the patient's tumor to process and present this peptide. These include immunoselection, HPV enhanced degradation of wt p53 molecules, a limited available T-cell repertoire, and tumor-induced apoptosis of anti-p53 CTL. Based on several lines of evidence, including the identification of genetic alterations of p53 as an early event in oral cancer, the immunoselection hypothesis is currently favored.

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Key Research Accomplishments

1. Identifying variant peptides capable of reversing the non-responsiveness of T lymphocytes to the wt $p53_{264\text{-}272}$ Epitope (14).

We sought to increase the responsive rate to the wt $p53_{264\text{-}272}$ peptide of PBMC obtained from normal donors and patients by identifying more immunogenic variants of

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[1]T.K. Hoffmann *et.al.*,Frequencies of tetramer+ T cells specific for the wild type sequence $p53_{264\text{-}272}$ peptide in the circulation of patients with head and neck cancer. *Submitted for publication,2001.*

this peptide. Two such variants were generated by amino acid exchanges at positions 6 (6T) and 7 (7W) of the peptide. These variants were capable of inducing T cells from PBMC of "non-responsive" donors that recognized the parental peptide either pulse onto target cells or naturally presented by tumors. TCR Vβ analysis of two T-cell lines isolated from bulk populations of effectors reactive against the wt $p53_{264-272}$ peptide, using either the parental or the 7W variant peptide, indicated that these T cells were [3]expressing identical TCR Vβ 13.6 / CDR3 / joining region sequences. This finding confirms the heteroclitic nature of at least one of the variant peptides identified in this study. The use of variant peptides of the wt $p53_{264-272}$ epitope represents a promising approach to overcoming the "non-responsiveness" of certain cancer patients to this "self" epitope, thereby enhancing its potential use in tumor vaccines for appropriately selected cancer patients.

2. Defining the Specificity of HLA-A2.1/wt $p53_{149-157}$ tetramer:

In addition to the wt $p53_{264-272}$ epitope, the wt $p53_{149-157}$ peptide was identified as an HLA-A2.1-restricted, CTL-defined wt p53 epitope. A $CD8^+$ anti-wt $p53_{149-157}$ specific CTL cell line induced by IVS of PBMC obtained from normal donors using autologous dendritic cells (DC) pulsed with the peptide acting as antigen presenting cells were used to characterize the specificity of a soluble PE-conjugated HLA-A2.1/wt $p53_{149-157}$ tetramer. The specificity of the anti-wt $p53_{149-157}$ CTL for the $p53_{149-157}$ peptide was determined in an ELISPOT IFNγ assays using peptide-pulsed T2 target cells, as indicated in **Table 1**. A control for this assay was the ant-$p53_{264-272}$ CTL line. As indicated in bold face in **Table 1**, both of these CTL lines were specific for the p53 peptide used to induce them. The the anti-$p53_{149-157}$ CTL cell line was then used in a 3- color flow cytometry analysis to define the specificity of the PE-conjugated HLA-A2.1/p53 $_{149-157}$ tetramer. The anti-$p53_{264-272}$ CTL line was an additional control for these studies, which also involved staining these cell lines with APC-conjugated HLA/A2.1 tetramers containing either the $p53_{65-73}$ or $p53_{217-225}$ peptide. The $p53_{149-157}$ tetramer recognized `50% of the CD3+ $CD8^+$ T cells in the cloned population of anti-$p53_{149-157}$ CTL, as shown in **Panel A,**

Figure 1. This tetramer binding inhibited anti-CD3 mAb binding to these T cells, an indication of tetramer specificity ( ). In contrast, the staining of this cell line with the other three p53 peptide tetramers did not show inhibition of anti-CD3 mAb binding. The specificity of HLA.A2.1/$p53_{149-157}$ tetramer was further confirmed by its inability to bind to anti-$p53_{264-272}$ CTL, which did bind the appropriate tetramer as shown in **Panel B, Figure 2**. Essentially the HLA-A2.1/$p53_{264-272}$ tetramer bound specifically to nearly all the T cells in this cell line. In this analysis, the irrelevant $p53_{65-73}$ shows a relatively high frequency of staining of both T cell lines, but this binding occurred in the absence of inhibition of binding of the anti-CD3 mAb and, therefore, is considered non-specific. As a result of this analysis, a second HLA-A2.1/p53 peptide tetramer is available for determining the frequency of anti-p53 precursor CTL in PBMC, as well as monitor cultures of in vitro restimulated PBMC for antip53 CTL.

3. Determing the frequencies of CD3+ CD8+ T cells recognizing HLA-A2.1-restricted wt p53 epitopes in clinical samples derived from breast cancer patients by flow cytometry analysis.

This retrospective study was limited to two sets of tumor-derived samples and/or blood obtained from breast cancer patients that were deposited in the UPCI Tissue and Tumor Bank: one set consisted of patients' PBMC, while the second set consisted of matched patients' samples of tumor infiltrating lymphocytes (TILs) and single cell suspensions of autologous breast tumor. The HLA haplotypes of these patients were unknown. Given that ~40% of patients are HLA-A2+, we anticipated that a similar percentage of the samples would be suitable for our study. Blood samples were sero-typed for HLA-A2 expression by flow cytometry analysis using two distinct mouse anti-HLA-A2 monoclonal antibodies, MA2.1 and BB7.2. Hybridomas producing both antibodies were obtained from American Type Culture Collection, Rockville, MD. Samples scoring positive with both antibodies were considered HLA-A2^{+}. The frequencies of anti-$p53_{149-157}$ and/or anti-$p53_{264-272}$ pCTL present in the two sets of samples, as determined by 4-color flow cytometry analysis, are reported in **Tables 2 and 3**, respectively. Approximately 2/3 of the patients' tested were HLA-A2^{+}. Again, only CD3+CD8+tetramer+ cells showing inhibition of binding of anti-CD3 mAb were

considered to represent specific binding events and are listed in bold face. **Table 3** also lists the results of the genomic analysis of p53 exons5-8 present in the autologous tumors.

As observed in our analysis of PBMC obtained from oral cancer patients, the frequencies of anti-$p53_{264-272}$ pCTL in PBMC and TILs fall into two groups: high vs low frequency of this pCTL (<1:1,000 vs >1:3,000). This may be an indication that breast cancer patients, like oral cancer patients, fall into two groups with respect to their responsiveness to p53 epitopes: responsive and non-responsive. The frequencies of anti-$p53_{264-272}$ pCTL in TIL samples is higher than observed in PBMC samples, as indicated in **Table 3**. . Although this analysis is limited in number, the higher frequencies of pCTL capable of recognizing a tumor antigen in TILs is consistent with the concept that anti-tumor effectors should be more readily found at tumor sites than in the general circulation. Finally, tumors of breast cancer patients showing high frequencies of anti-$p53_{264-272}$ pCTL in TIL samples , e.g., patient # 6294 and #6295, express altered p53 molecules and, therefore, have a reasonable potential to present this epitope. Although the trends observed in this aspect of our study are limited due to the relatively small number of samples analyzed, they are supportive of clinical use of p53-based vaccines for immunotherapy of breast cancers, and definitely warrant further investigation.

Reportable Outcomes

Publications:

T. K. Hoffmann, D. J. Loftus, K. Nakano, M. J. Maeurer, K. Chikamatsu, E. Appella, T. L. Whiteside and A. B. DeLeo. The Ability of Variant Peptides to Reverse the Non-responsiveness of T Lymphocytes to the Wild-Type Sequence $p53_{264-272}$ Epitope. *J.Immunol. in press*, 2002.

Conclusions

1) Identified the use of two altered peptide ligands to reverse the non-responsiveness of anti-$p53_{264-272}$ pCTL present in PBMC of cancer patients, many of whose tumors have the potential to present this epitope for T-cell recognition
2) The specificity of the HLA-A2.1/$p53_{149-157}$ tetramer was confirmed and a protocol

for its use in detecting anti-$p53_{149-157}$ pCTL in PBMC established. The pCTL recognizing this p53 peptide appeared to be present in these samples at lower frequencies than the anti-$p53_{264-272}$ pCTL.

3) As observed in our analysis of PBMC obtained from oral cancer patients, the frequency of anti-$p53_{264-272}$ pCTL in PBMC of breast cancer patients appears to define two subsets of patients: high vs low frequency of this pCTL. This may be an indication that breast cancer patients, like oral cancer patients, fall into two groups with respect to antipp53 responsiveness: responsive and non-responsive.
4) The frequencies of anti-$p53_{264-272}$ pCTL in TIL samples is higher than observed in PBMC samples. Although this analysis is limited in numbers, the higher frequencies of pCTL capable of recognizing a tumor antigen in TILs is what many tumor immunologists expect to be observed.
5) Tumors of breast cancer patients showing high frequencies of anti-$p53_{264-272}$ pCTL in TIL samples have a reasonable potential to present this epitope and is supportive of clinical use of p53-based vaccines for immunotherapy of breast cancers.

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Appendices

T. K. Hoffman *et.al., J. Immunol. in press* (2002).

Table 1: Specificities of bulk populations of anti-wt p53 CTL generated by IVS of PBMC obtained from HLA-A2^{+} normal donors using autologous dendritic cells pulsed with wt p53 peptides: Analysis using ELISPOT IFNγ assays

IVS with peptide	Spots/10^4 T cells peptide pulsed-T2 target cells		
	None	$p53_{149-157}$	$p53_{264-272}$
$p53_{149-157}$	16.3±1.5	312 3±52.1	13.0±7.9
$p53_{264-272}$	26.3±3.8	9.0±7.9	630.0±36.4

Table 2. Frequency anti-wt p53 pCTL detected in $CD3^+CD8^+$ T-cells in PBMC obtained from HLA-$A2^+$ breast cancer patients[a]

	Frequencies of CD3+CD8+tetramer+ cells[a]	
	Anti-wt $p53_{264-272}$	anti-wt $p53_{149-157}$
HLA-$A2^+$ donors		
Patients		
#88517	**1:150**	**nd**
#86027	**1:222**	1:2,893
#89692	**1:2,899**	1:11,960
#88069	**1:3,977**	**nd**
Normal donor		
#86651	**1:581**	**1:10,956**
HLA-$A2^-$ Donors		
Patients		
#86319	>1:25,000	**1:8,359 / 10**
#91757	1:18,543	**nd**
Normal donor		
#88027	1:6,827	**nd**

[a] Frequencies listed in bold face denotes inhibition of anti-CD3 mAb binding to $CD3^+CD8^+tetramer^+$ cells and signifies specificity of tetramer binding ( ).

Table 3 . Flow cytometry analysis of frequencies of anti-wt $p53_{264-272}$ pCTL detected in $CD3^+CD8^+$ T-cells in TILs obtained from breast cancer patients using HLA-A2.1/$p53_{264-272}$ tetramer

pCTL[b]	p53 status[a]	Frequency of anti-$p53_{264-272}$
HLA-$A2^+$ patients		
#1216	**wt**	**1:1,226**
#6294	**R248G (exon 7)**	**1:54**
#7418	**G266H exon 7)**	**insufficient T cells**
#5281	**wt**	**1:13,756**
#6495	**exon 6 deletion**	**1:171**
#3001	**nd**	**1:11,104**
#0827	**nd**	**1:1,626**
HLA-$A2^-$ patients		
#0226	**wt**	1:10,750

[a] These analyses do not exclude point mutational damage outside exons 5-8 which was not within the scope of this analysis.

[b] Frequencies listed in Bold face denotes inhibition of αCD3 mAb binding to $CD3^+CD8^+$tetramer$^+$ cells and signifies specificity of tetramer binding ( ).

Figure 1. Specificity of binding of $p53_{149\text{-}157}$ and $p53_{264\text{-}272}$ peptide/HLA-A2.1 tetrameric complexes against cloned anti-p53 peptide CD8+ T cell lines by three-color flow cytometry

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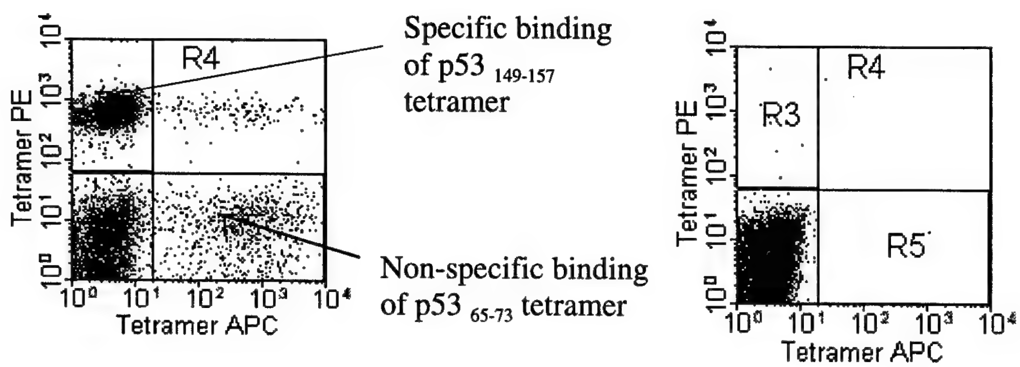
Panel A: Analysis of anti-$p53_{149\text{-}157}$ CD8+ T cells with p53 peptide tetramers shows that only the $p53_{149\text{-}157}$ peptide tetramer shows specificity: high binding with inhibition of anti-CD3 mAb binding.



$p53_{149\text{-}157}$ PE and $p53_{65\text{-}73}$.APC peptide tetramers

p53 $_{217\text{-}225}$ APC and $p53_{264\text{-}272}$ PE peptide tetramers

Tetramer:frequency:anti-CD3 mAb inhibition

149 PE: 1:2; yes

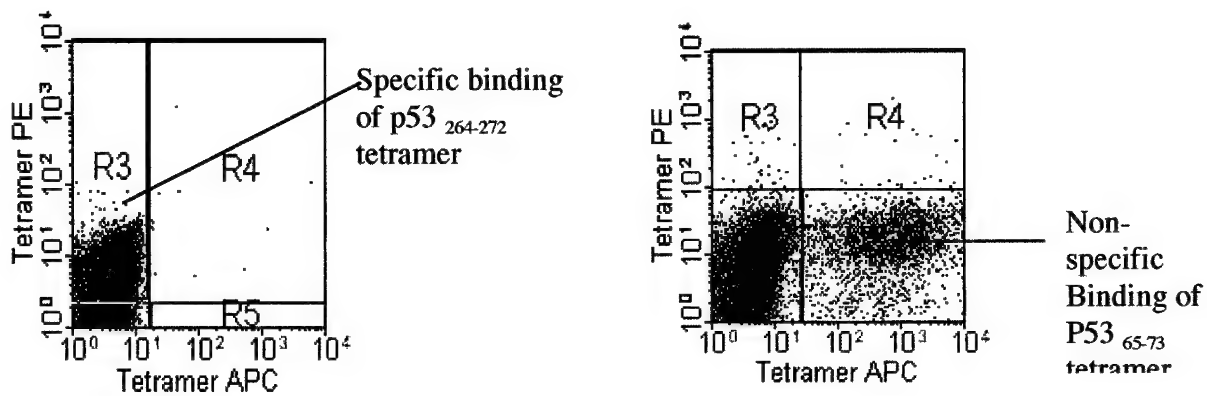
65APC: 1:5.5; none

217-APC: 1:1,824;none

264-PE: 1:839:none

Figure 1:

Panel B: Analysis of anti-p53$_{264\text{-}272}$ CD8+ T cells with p53 peptide tetramers shows that only the p53 $_{64\text{-}2727}$ peptide tetramer shows specificity: high binding with CD3 shift



p53$_{149\text{-}157}$ PE and
p53$_{65\text{-}73}$.APC peptide tetramers

p53 $_{217\text{-}225}$ APC +
p53 $_{264\text{-}272}$ PE peptide tetramers

Tetramer:frequency:anti-CD3 mAb inhibition

217-APC: 1:3153; none

149-PE: 1:253; none

264-PE:1:1: yes

65-APC: 1:4; none

The Ability of Variant Peptides to Reverse the Nonresponsiveness of T Lymphocytes to the Wild-Type Sequence $p53_{264-272}$ Epitope[1]

Thomas K. Hoffmann,* Douglas J. Loftus,† Koji Nakano,* Markus J. Maeurer,‡ Kazuaki Chikamatsu,* Ettore Appella,† Theresa L. Whiteside,*§¶ and Albert B. DeLeo[2]*§

Recently, we observed that CTL specific for the wild-type (wt) sequence $p53_{264-272}$ peptide could only be expanded ex vivo from PBMC of a subset of the HLA-A2.1$^+$ normal donors or cancer patients tested. Surprisingly, the tumors of the responsive patients expressed normal levels of wt p53 and could be considered unlikely to present this epitope. In contrast, tumors of nonresponsive patients accumulated mutant p53 and were more likely to present this epitope. We sought to increase the responsive rate to the wt $p53_{264-272}$ peptide of PBMC obtained from normal donors and patients by identifying more immunogenic variants of this peptide. Two such variants were generated by amino acid exchanges at positions 6 (6T) and 7 (7W) of the peptide. These variants were capable of inducing T cells from PBMC of nonresponsive donors that recognized the parental peptide either pulsed onto target cells or naturally presented by tumors. TCR Vβ analysis of two T cell lines isolated from bulk populations of effectors reactive against the wt $p53_{264-272}$ peptide, using either the parental or the 7W variant peptide, indicated that these T cells were expressing identical TCR Vβ13.6/complementarity-determining region 3/J region sequences. This finding confirms the heteroclitic nature of at least one of the variant peptides identified in this study. The use of variant peptides of the wt $p53_{264-272}$ epitope represents a promising approach to overcoming the nonresponsiveness of certain cancer patients to this self epitope, thereby enhancing its potential use in tumor vaccines for appropriately selected cancer patients. *The Journal of Immunology,* **2002, 168: 0000–0000.**

Genetic alterations in p53 occur in a wide range of human tumors, including oral squamous cell carcinomas (OSCC)[3] (1). The most common type of genetic alteration in p53 involves a missense mutation that is usually accompanied by accumulation of the altered molecules in the cytosol of tumor cells. Initially, the effort to develop p53-based vaccines focused on these missense mutations, which are tumor specific in nature. However, missense mutations have limited clinical usefulness, because of the requirement that they occur within or create epitopes that could be presented by MHC molecules expressed by the individual patient. On the other hand, the majority of p53 epitopes derived from these altered p53 molecules would be wild type in sequence, representing a new class of tumor-associated self Ags that are candidates for use in the development of broadly applicable cancer vaccines (1–5).

To date, five MHC class I-restricted, naturally presented human wild-type (wt) sequence p53 epitopes have been identified. They have been shown to be able to induce epitope-specific CTL from PBMC obtained from healthy individuals (1, 6–11). The $p53_{125-134}$ epitope is HLA-A24 restricted (11), while the other four, $p53_{65-73}$, $p53_{149-157}$, $p53_{217-225}$, and $p53_{264-272}$, are HLA-A2.1 restricted. Among these, the wt $p53_{264-272}$ peptide has been the most intensively investigated (1, 2, 6–8, 12).

The potential of wt p53 epitopes as targets for immunotherapy, however, remains uncertain due to the several critical concerns related to immunological recognition of this truly self tumor Ag. Using HLA-2.1-transgenic wt ($p53^{+/+}$) and $p53^{null}$ ($p53^{-/-}$) mice, Sherman and colleagues (13–15) have demonstrated that the CTL repertoire available for wt p53 self epitopes in $p53^{+/+}$ mice is limited to intermediate affinity T cells, because the higher affinity CTL are either deleted or tolerized. Apparently, this situation occurs in humans as well, as only CTL with intermediate affinity for the wt $p53_{264-272}$ epitope have been generated to date from PBMC obtained from normal donors as well as cancer patients (7, 12). This observation raises the question of whether such CTL are potent enough to be effective in tumor eradication. AQ: A

Another concern relates to our experience that PBMC obtained from only some HLA-A2.1$^+$ healthy donors and patients with OSCC were responsive to in vitro stimulation (IVS) with the wt $p53_{264-272}$ peptide pulsed onto autologous dendritic cells (DC) (7, 12). Furthermore, CTL reactive against this epitope could only be generated from T cell precursors in PBMC of patients whose tumors were not likely to present this epitope. The analysis of these tumors indicated no accumulation of p53 or accumulation of mutant p53 with a missense mutation at codon 273, a site known to

*University of Pittsburgh Cancer Institute and Departments of §Pathology and ¶Otolaryngology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213; †National Cancer Institute, Bethesda, MD; and ‡Department of Medical Microbiology, Johannes Gutenberg University, Mainz, Germany AQ: G

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[2] Address correspondence and reprint requests to Dr. Albert B. DeLeo, University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Division of Basic Research, Biomedical Science Tower W956, 211 Lothrop Street, Pittsburgh, PA 15213. E-mail address: deleo@imap.pitt.edu

AQ: J [3] Abbreviations used in this paper: OSCC, oral squamous cell carcinoma; APL, altered peptide ligand; CDR, complementarity-determining region; DC, dendritic cell; IVS, in vitro stimulation; wt, wild type.

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block processing of the wt $p53_{264-272}$ epitope (16). In contrast, PBMC obtained from patients with tumors considered capable of presenting the wt $p53_{264-272}$ epitope (i.e., tumors that accumulate mutant p53) were nonresponsive to IVS with wt $p53_{264-272}$-pulsed autologous DC. These findings have led us to conclude that CTL specific for the wt $p53_{264-272}$ epitope might play a role in the outgrowth of epitope-loss tumor cells, which are able to escape from the host immune system. This conclusion was further strengthened by the results of a recently completed study in our laboratories that used tetrameric peptide/MHC class I complexes to determine frequencies and characteristics of the $p53_{264-272}$-specific CTL in unstimulated PBMC obtained from 30 OSCC patients and 31 normal donors (data not shown) (2).

Because these observations suggest that it may be possible to accurately predict ex vivo the responsiveness of cancer patients to immunotherapy targeting this epitope, we felt that a means of circumventing the nonresponsiveness of individuals needed to be investigated to proceed with the development of wt p53-based vaccines. One solution is to identify a heteroclitic peptide or, in more precise terms, an altered peptide ligand (APL) with enhanced functional activity relative to the parental wt $p53_{264-272}$ peptide. By substituting amino acids at various positions of an epitope that contact MHC class I, and/or TCR, an array of APL with biological potencies higher than those of the parental epitopes has been identified for various antigenic determinants (17–26). In applying this strategy to the wt $p53_{264-272}$ epitope, we anticipated that an APL might induce CTL-mediated responses that cross-react with the parental epitope and that these CTLs also might demonstrate enhanced avidities relative to CTLs induced by the parental peptide. Most importantly, we sought to determine whether an APL would be able to induce anti-wt $p53_{264-272}$ CTL from PBMC that were nonresponsive to the parental peptide, particularly the PBMC obtained from patients whose tumors accumulate mutant p53 and are considered to have the potential to present this epitope.

Materials and Methods

Cell lines and cell culture

The following HLA-A2^{+} OSCC cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA): SCC-4 and SCC-9. The SCC-4 cell line expresses and accumulates p53 expressing a missense mutation at codon 151 but does not present the wt $p53_{264-272}$ epitope (6). The SCC-9 cell line expresses an altered p53 molecule with a deletion of codons 274–285. It does not accumulate p53 molecules, yet presents the wt $p53_{264-272}$ epitope. In addition, the $p53^{null}$ osteosarcoma cell line, SaOS-2, was obtained from ATCC. The cloned $p53^{+}$ cell line, SaOS-2C13, was derived by transduction of SaOS-2 cells with a p53 cDNA expressing a missense mutation in codon 143 (7). The HLA-A2^{+} OSCC cell line PCI-13 has been described previously (27). It expresses a p53 missense mutation in codon 286 (Glu to Lys) and presents the wt $p53_{264-272}$ epitope. Tumor cells were cultured in plastic culture flasks (Costar, Cambridge, CA) under standard conditions (37°C, 5% CO_2 in a fully humidified atmosphere) in complete medium consisting of DMEM (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 IU/ml penicillin (all from Life Technologies). The T2 cell line was also obtained from ATCC and maintained in RPMI 1640 (Life Technologies) containing 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics. The cultures were routinely tested and found to be free of mycoplasma contamination (Gen-Probe, San Diego, CA).

Peptides

The CTL-defined, HLA-A2.1-binding peptide, LLGRNSFEV (1), corresponding to wt $p53_{264-272}$, as well as single amino acid exchange variants of this peptide were synthesized by standard *N*-(9-fluorenyl)methoxycarbonyl methology. Peptides were purified by reversed-phase HPLC, and their amino acid sequence was confirmed by mass spectrometry analysis. All peptides were dissolved in DMSO (Fisher Scientific, Pittsburgh, PA) at 1 mg/ml and diluted with PBS just before use. The 19 variant peptides contain single amino acid exchanges with a bias toward retention of a high degree of similarity to the central region of the parental peptide. The variant peptides are designated 1E, 1F, 1V, 3L, 3F, 3W, 4K, 4L, 5K, 5L, 6G, 6T, 6Y, 7L, 7P, 7Y, 7W, 8A, and 8Y, in which numbers denote the position within the parental sequence and letters refer to exchanged amino acids.

MHC stabilization assay

T2 cells were incubated overnight at room temperature before use in this assay. Cells were washed and incubated at a cell density of 2×10^5/0.2 ml of complete medium with various peptides at final concentrations of 1×10^{-5}–1×10^{-10} M for 3 h at room temperature, followed by a 3-h incubation period at 37°C. After washing with PBS, cells were incubated at 4°C for 30 min with anti-HLA class I mAb, W6/32 (HB95; ATCC), and then with FITC-conjugated goat anti-mouse Ig (Caltag Laboratories, Burlingame, CA) as a secondary Ab. Fluorescence of viable T2 cells was measured at 488 nm in a FACScan flow cytometer (BD Biosciences, San Jose, CA), and the level of MHC class I expression was determined by evaluating the mean fluorescence intensity of stained T2 cells. Cells incubated either at room temperature or 37°C in the absence of peptide served as controls. AQ: B

Generation of anti-p53 CTL with peptide-pulsed autologous DC

Peripheral blood or leukapheresis products were obtained from previously studied HLA-A2.1^{+} individuals: seven normal donors and six OSCC patients (12). PBMC were isolated by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ). The study was approved by the Institutional Review Board at the University of Pittsburgh, and written informed consent was obtained from each individual donating peripheral blood. PBMC were phenotyped for HLA-A2 expression by flow cytometry, using anti-HLA-A2 mAb, BB7.2 (HB82; ATCC), and a mouse IgG isotype as a control. The verification of the A0201 subtype was performed using PCR with sequence-specific primers, as previously described (12).

Human DC were generated from PBMC according to a modification of the method of Sallusto and Lanzavecchia (28), as described by us earlier (7). DC were harvested on day 6, phenotyped by flow cytometry, and then resuspended in AIM-V medium (2×10^6 cells/ml) containing 10 μg/ml peptide and incubated at 37°C for 4 h. The peptide-pulsed DC were then cocultured with autologous PBMC in 24-well tissue culture plates (Costar) in a final volume of 2 ml/well AIM-V medium supplemented with 10% (v/v) human AB serum (Pel-Freez Biologicals, Brown Deer, WI) and 25 ng/ml IL-7 (Genzyme, Cambridge, MA) for the first 72 h and, additionally, with 20 IU/ml IL-2 (Chiron-Cetus, Emeryville, CA) for the remaining time in culture. The lymphocytes were restimulated 1 wk later with peptide-pulsed autologous DC. Irradiated (3000 rad) autologous PBMC were used as APC after the third round of restimulations. Microcultures of CTL lines recognizing the wt $p53_{264-272}$ or 7W peptide were isolated from bulk populations of effectors by limiting dilution (1 cell/well/96-well plates), and the lines were maintained in cytokine-supplemented media plus peptide-pulsed APC, as previously described (7). Specificities of generated T cells were determined using one or more of a panel of assays detailed below. The TCR Vβ expression on T cells in bulk CTL populations and cell lines derived from them was done using the IOTest β Mark TCR Vβ Repertoire kit (Beckman Coulter, San Diego, CA). AQ: C

ELISPOT assay for IFN-γ

The ELISPOT assay was performed in 96-well plates with nitrocellulose membrane inserts (Millipore, Bedford, MA), as previously described by us (29). The capture and detection anti-IFN-γ mAb were purchased from Mabtech (Nacka, Sweden). The spots were counted by computer-assisted image analysis (ELISPOT 4.14.3; Zeiss, Jena, Germany). For Ab-blocking experiments, target cells were preincubated with anti-HLA class I mAb for 30 min. Cryopreserved aliquots of PBMC obtained from a normal donor were thawed and, after stimulation with PMA (1 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich, St. Louis, MO), were used as a positive control for each assay. The interassay reproducibility of the assay was acceptable with a CV = 15% (n = 30). AQ: D

Cytotoxicity assay

The 4-h ^{51}Cr release assay was performed at various E:T ratios, as previously described (7). Briefly, sensitized targets were labeled with ^{51}Cr for 45 min at 37°C, washed, and added to wells of 96-well plates (1×10^4 cells/well). Effector T cells were then added to give various E:T ratios. When Ab-blocking experiments were performed, target cells were incubated with anti-HLA class I mAb or the anti-HLA-DR mAb, L243 (HB55; ATCC),

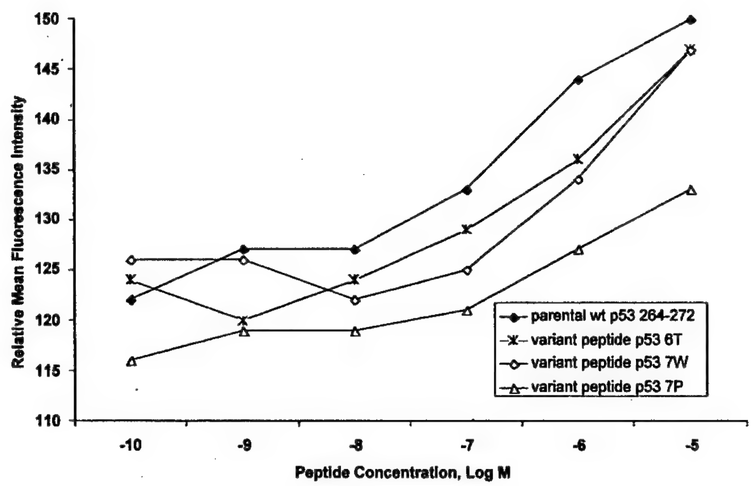

FIGURE 1. Identification of three HLA-A2.1-binding variant peptides of the wt $p53_{264-272}$ epitope. T2 cells were incubated with parental $p53_{264-272}$ peptide (LLGRNSFEV) or 6T, 7P, or 7W variant peptides at final concentrations of 1×10^{-5}–1×10^{-10} M. The relative mean fluorescence intensities of FITC-conjugated anti-MHC class I mAb (W6/32) are indicative of peptide-stabilized MHC class I molecules on T2 cells.

for 30 min before adding effector cells. The percentage of specific lysis was calculated according to the formula:

$$\% \text{ Specific lysis} = \frac{\text{Experimental cpm} - \text{control cpm}}{\text{Maximal cpm} - \text{control cpm}} \times 100$$

Flow cytometry analysis using HLA-A2.1/peptide tetrameric complexes (tetramer)

The streptavidin-PE-labeled tetramers used in this study were obtained from the tetramer core facility of the National Institute of Allergy and Infectious Disease (Atlanta, GA). Three-color flow cytometry assays (FACScan; BD Biosciences) were performed with PerCP anti-CD3, FITC anti-CD8, and PE-tetramer. The specificity of the HLA-A2.1/$p53_{264-272}$ tetramer was confirmed by its staining of a CTL line specific for this p53 epitope and by the lack of staining of irrelevant CTL or HLA-A2$^-$ PBMC of healthy donors, as previously described (30). The additional PE-conjugated HLA-A2.1/tetramer used in this study contained the 7W variant peptide. Generally, 75,000 events per sample were collected progressively after live gating on lymphocytes by forward and side scatter.

TCR and CDR3 spectratyping

RNA was extracted from $p53_{264-272}$-specific CTL lines generated using parental or the 7W variant peptide, followed by reverse transcription into cDNA, as previously described (31). Screening for expression of TCR V
AQ: E chains was performed using the primers described by Puisieux et al. (32) for TCR Vβ amplification, followed by a runoff reaction with fluorophore-labeled primers specific for the C region of the TCRβ (5′-TGTGCAC CTCCTTCCCATTCACC) chain. Labeled runoff products were subjected to DNA fragment analysis, as described (32). Finally, amplified products were directly subjected to DNA sequence analysis using ABI 310 sequencer (PerkinElmer, Weiterstadt, Germany).

Statistical analysis

A two-tailed Wilcoxon rank sum test was performed to analyze ELISPOT data. An unpaired two-tailed Student's *t* test was used to interpret differences in CTL reactivities against different target cells and in the presence of blocking Ab in cytotoxic assays, and differences between the number of spots obtained from T cells incubated with T2 cells pulsed with relevant p53 peptides vs that obtained using T2 cells pulsed with the irrelevant gp100 peptide in ELISPOT assays. Differences were considered significant when $p < 0.05$.

Results

Selection of variant $p53_{264-272}$ peptides recognized by anti-$p53_{264-272}$ CTL

Because the parental peptide binds efficiently to HLA-A2.1 molecules, all of the APL considered in this study represent single amino acid exchanges at nonanchor residues for the purpose of enhancing the interactions of the variant peptides with the TCR

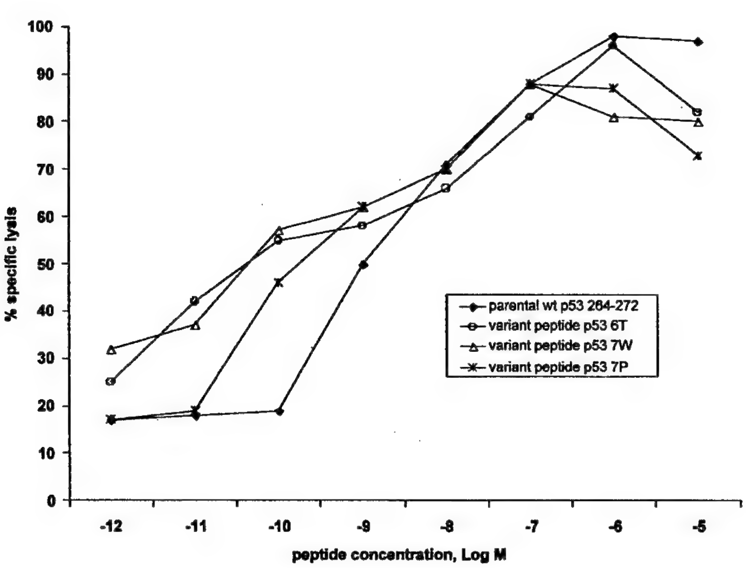

FIGURE 2. Variant peptides are recognized by anti-wt $p53_{264-272}$-specific CTL line. T2 cells were pulsed with different peptide concentrations and tested as targets in a 4-h ^{51}Cr release assay at the E:T ratio of 10:1.

rather than MHC class I molecules. Nineteen variants of the wt $p53_{264-272}$ peptide were screened for their recognition by a bulk population of anti-wt $p53_{264-274}$-specific CTL that was maintained in our laboratory (7). T2 cells pulsed with the individual peptides at a fixed concentration of 1×10^{-6} M peptide served as targets for these CTL in a ^{51}Cr release cytotoxic assay. Significant cytotoxic reactivity against T2 cells pulsed with three of the 19 variant peptides, namely, 6T, 7W, and 7P, was detected (data not shown). Therefore, these three variant peptides were selected for further characterization.

Variant peptide binding to HLA-A2.1 molecules

Binding of the 6T, 7W, and 7P variant peptides to HLA-A2.1 molecules was compared with that of the parental peptide in an MHC stabilization assay. The relative mean fluorescence intensity of parental and variant peptide-stabilized HLA-A2 molecules on
F1 T2 cells is shown in Fig. 1. All the peptides showed stabilization of HLA-A2 molecules in a dose-dependent manner within the concentration range of 1×10^{-5}–1×10^{-9} M. However, in general, the binding affinities of the variant peptides to HLA-A2.1 molecules on T2 cells were slightly lower than that of the parental wt peptide (wt > 6T ≥ 7W > 7P).

Affinity of $p53_{264-272}$-specific CTL for variant peptides

The affinity of the bulk population of anti-$p53_{264-272}$-specific CTL for the variant peptides was determined in a 4-h ^{51}Cr release assay using T2 cells pulsed with these peptides at concentrations ranging from 1×10^{-5} to 1×10^{-12} M as target cells. As shown in Fig.
F2 2, at concentrations $<1 \times 10^{-8}$ M, the dose-response curves of the three variant peptides were shifted to the left relative to that of the parental wt peptide. Because the increased responsiveness of the CTL for these variant peptides cannot be attributed to enhanced binding to HLA-A2.1 molecules, these results are consistent with an increased affinity of TCR for the variant peptides.

Characterization of wt $p53_{264-272}$-specific CTL generated from PBMC obtained from normal donors using variant peptides

Previously, we reported that CTL reactive against the wt $p53_{264-272}$ epitope could be generated from PBMC obtained from only two of the seven HLA-A2.1^{+} normal donors tested (12). Analyses involving multiple cryopreserved samples derived from leukopaks obtained from two of the normal donors (a responder and a nonresponder) confirmed the consistency of responses of these donors' PBMC to the parental peptide. In the same experiments in which the seven donors' PBMC were tested for induction using the parental peptide, CTL reactive against this peptide could be generated from five of these seven PBMC using either the 6T or 7W variant peptide. Included in this group were three nonresponsive PBMC (Table I); PBMC obtained from donors 6 and 7 responded T1
to the 7W variant, while PBMC obtained from donor 4 responded to the 6T peptide. None of the seven PBMC tested responded to the 7P variant peptide.

The bulk populations of variant-induced cells generated from PBMC obtained from donors 6 and 7 effectively recognized and lysed T2 cells pulsed with the parental peptide in ELISPOT for IFN-γ and cytotoxicity assays. Fig. 3, *A* and *B*, shows the results F3

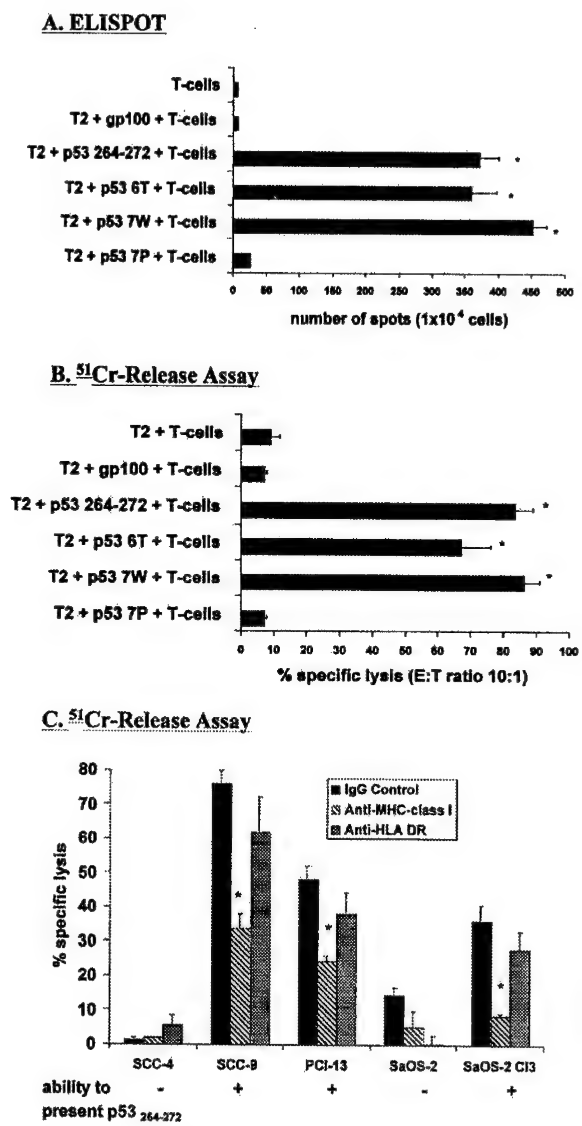

FIGURE 3. The 7W variant peptide-induced effectors induced from PBMC obtained from a nonresponsive healthy donor recognize the parental wt $p53_{264-272}$ peptide pulsed onto target cells or naturally presented by tumors. *A*, Recognition of peptide-pulsed T2 cells in ELISPOT for IFN-γ assays. Effectors were tested against T2 cells pulsed with an irrelevant gp100 peptide, the wt $p53_{264-272}$ peptide, or the variant peptides at 10 μg/ml. *B*, Lysis of T2 cells pulsed with various peptides at an E:T of 1:10. *C*, Lysis of tumor targets naturally presenting the epitope at an E:T ratio of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. *, A significant ($p < 0.05$) difference relative to IgG controls.

Table I. *Summary of the anti-p53 CTL responses of PBMC obtained from normal donors following IVS using variant $p53_{264-272}$ peptides*[a]

	Anti-p53 CTL Response after IVS With			
Donor	wt $p53_{264-272}$	Variant 6T	Variant 7P	Variant 7W
1	+	−	−	+
2	+	−	−	+
3	−	−	−	−
4	−	+	−	−
5	−	−	−	−
6	−	−	−	+
7	−	−	−	+

[a] PBMC were stimulated with the peptide-pulsed autologous DC or PBMC in two to four IVS cycles. Effector cell reactivity was tested in ELISPOT and cytotoxicity assays; − indicates that no specific reactivity against the wt $p53_{264-272}$ peptide was observed, while + indicates that effectors were reactive against variant and parental peptides. Results using the parental wt $p53_{264-272}$ were reported in a previous publication from this laboratory (12).

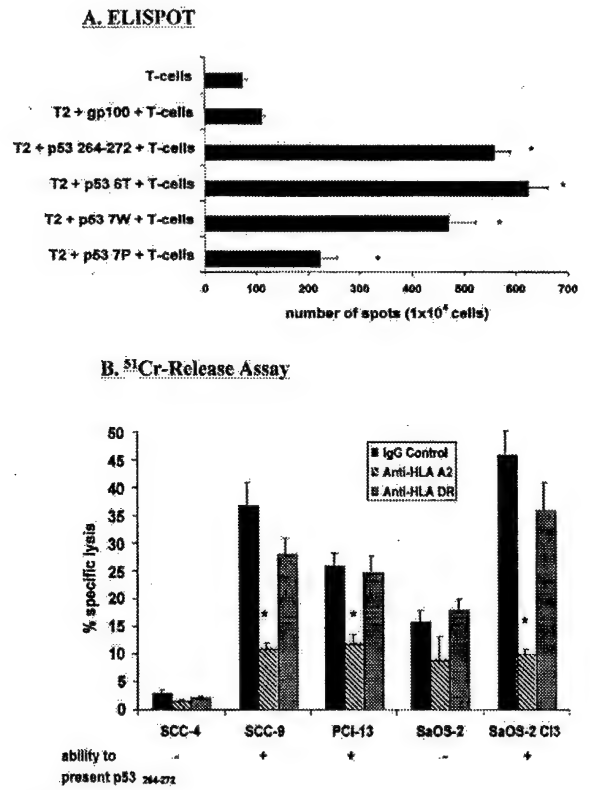

FIGURE 4. The 6T variant peptide-induced effectors induced from PBMC obtained from nonresponsive healthy donor recognize the parental wt $p53_{264-272}$ epitope pulsed onto target cells or naturally presented by tumors. *A*, Recognition of peptide-pulsed T2 cells in ELISPOT assays. Effectors were tested against T2 cells pulsed with either an irrelevant gp100 peptide, the wt $p53_{264-272}$ peptide, or a variant peptide at 10 μg/ml. *B*, Lysis of tumor targets naturally presenting the epitope at an E:T of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. *, A significant ($p < 0.05$) difference relative to IgG controls.

obtained with the effectors generated from PBMC obtained from donor 7. Unpulsed T2 target cells or T2 cells pulsed with an irrelevant HLA-A2.1-binding peptide, the melanoma-associated gp100 peptide (33), were not recognized by these CTL in either assay to any noticeable extent. More importantly, these effector cells were also capable of recognizing the naturally presented epitope, as evidenced by their ability to lyse PCI-13 and SCC-9 tumor cells as well as mutant p53-transfected SaOS-2 Cl3 cell lines in a MHC class I-restricted manner (Fig. 3*C*). No significant cytotoxicity was noted against HLA-A2$^+$ tumor cell lines, SCC-4 and SaOS-2, which do not present the epitope.

The CTL generated from PBMC obtained from donor 4 using the 6T peptide yielded effectors with reactivity comparable with that of the 7W variant-induced CTL. The 6T-induced T cells were responsive to wt $p53_{264-272}$-pulsed T2 cells in the ELISPOT for IFN-γ assay (Fig. 4*A*), and cytolytic against the OSCC lines, SCC-9 and PCI-13, as well as SaOS-2Cl3 (Fig. 4*B*). This response was blocked by anti-HLA class I mAb but not anti-HLA-DR mAb. No significant reactivity was obtained against the tumor cell line SCC-4. The reactivity of these effectors against SaOS-2 cells in the analysis shown in Fig. 4*B* was higher than normally detected against this $p53^{null}$ cell line, using bulk populations of anti-p53 effectors (7, 12). However, the reactivity of the 6T-induced effectors against SaOS-2 targets was not significantly blocked by anti-HLA class I mAb and thus could be attributed to nonspecific effectors present in the bulk population. In summary, variant-induced effector T cells had similar reactivities against the parental epitope as those reported previously for the parental peptide-induced effectors from responsive normal donors as well as OSCC patients (7, 12). F4

Characterization of wt $p53_{264-272}$-specific CTL generated from PBMC of a nonresponsive OSCC patient using a variant peptide

The critical test of the variant peptides was whether their use could induce CTL capable of recognizing the anti-wt $p53_{264-272}$ epitope from nonresponsive patients whose tumors were considered capable of presenting this epitope (12). The nonresponsiveness of PBMC obtained from at least one of these donors, patient 3, has been repeatedly confirmed during the past 2 years using blood samples obtained at different times, as well as multiple cryopreserved leukapheresis samples obtained from this patient. As shown in Table II, none of the PBMC from three of these patients responded to the 6T or 7P variant peptides. However, the 7W variant peptide did induce the ex vivo generation of anti-wt $p53_{264-272}$ CTL from PBMC of patient 3, whose autologous tumor, PCI-13, presents this epitope (7, 12). The affinity of these effectors for the parental epitope was comparable with that of a bulk population of CTL induced using the parental epitope, in the range of 1×10^{-9} M (Figs. 2 and 5*A*). Furthermore, the 7W-induced CTL were cytotoxic against a panel of tumor cell lines naturally presenting the wt $p53_{264-272}$ epitope, including the autologous PCI-13 cell line, and this reactivity was MHC class I restricted (Fig. 5*B*). This result clearly illustrates the potential value of the 7W variant peptide in immunotherapy targeting the wt $p53_{264-272}$ epitope in individuals like OSCC patient 3. T2 F5

Table II. *Summary of the anti-p53 CTL responses of PBMC obtained from nonresponsive OSCC patients following IVS using variant $p53_{264-272}$ peptides*

Patient	Tumor[a]		Anti-p53 CTL Response After IVS[b]		
	p53 genotype	p53 protein	Variant 6T	Variant 7P	Variant 7W
1	Mutant R248W	+	−	−	−
2	Mutant V 157 F	+	−	−	−
3	Mutant E286K	+	−	−	+

[a] Patients' tumors were analyzed for genetic alterations in p53 exons 5–8, and the identified codon and missense mutations are denoted. The level of p53 expression in tumors was determined by immunohistochemistry, using anti-p53 mAb, and + denotes accumulation of p53 (12).

[b] PBMC were stimulated with the peptide-pulsed autologous DC or PBMC in two to four IVS cycles. Effector cell reactivity was tested in ELISPOT and cytotoxicity assays; − indicates that no specific reactivity against the wt $p53_{264-272}$ peptide was observed, while + indicates that effectors were reactive against variant and parental peptides.

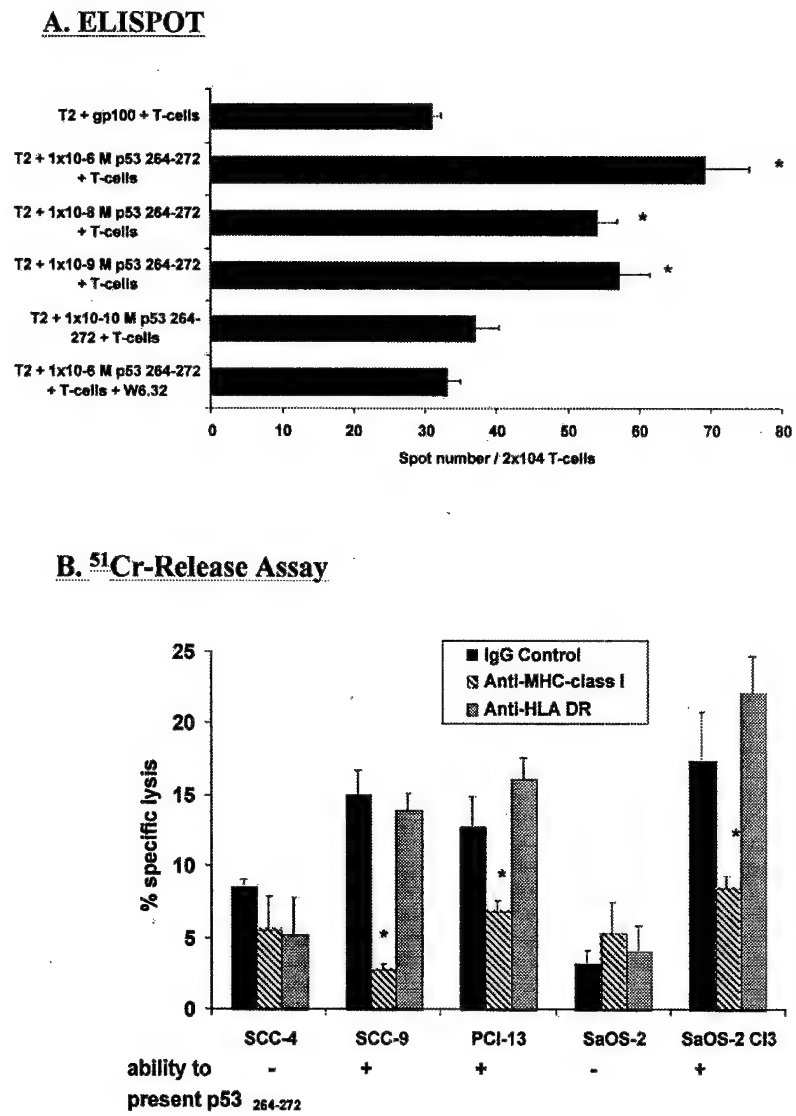

FIGURE 5. The 7W variant peptide-induced effectors obtained from a nonresponsive OSCC patient recognize the parental wt $p53_{264-272}$ peptide pulsed onto target cells or naturally presented by tumors, including PCI-13, the autologous tumor cell line. *A*, Affinity of effectors for the parental wt $p53_{264-272}$ peptide as determined in ELISPOT assay. T2 cells were incubated with parental peptide at concentrations of 1×10^{-6}–1×10^{-10} M. T2 cells pulsed with an irrelevant gp100 peptide served as a control. *B*, Lysis of tumor targets naturally presenting the epitope at an E:T of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. *, A significant ($p < 0.05$) difference relative to IgG controls.

Tetramer-binding and TCR Vβ usage by T cell microcultures reactive against parental and/or variant peptides

The ability of the variant peptides to induce the generation of CTL specific for wt $p53_{264-272}$ from nonresponder PBMC raised the question of the relationship between these CTL and those induced by the parental peptide in responder PBMC. The need to investigate this relationship became evident when the cross-reactive bulk population of $CD8^+$ T cells induced with the 7W variant peptide from normal donor 7 was stained with the parental or variant tetramer. Whereas only ~2% tetramer^{dim} cells were detected with the parental tetramer, a cluster of ~40% tetramer^{bright} cells was de-
F6 tected with the 7W tetramer (Fig. 6, *A* and *B*). One possible explanation for this observed difference was that the variant peptide induced a single $CD8^+$ T cell population that bound the variant tetramer with higher avidity/stability than did the parental tetramer. Another possible explanation was that the variant peptide induced two distinct populations of $CD8^+$ T cells; one was cross-reactive and bound both tetramers (most likely with different avidities), while the other was specific for the 7W variant and bound the 7W tetramer with high avidity. The two possibilities could be distinguished based on TCR usage of the T cells involved in recognition of these peptides. To accomplish this, T cell microcultures were established by limiting dilution from bulk CTL populations induced with either parental or variant peptide. Several T cell clones from each type of microculture were expanded for further analysis. Based in part on their rates of proliferation as well as peptide specificities (Fig. 7), four oligoclonal T cell lines, desig- F7
nated 2, 4, 53, and 68, were selected for TCR analysis by complementarity-determining region (CDR)3 spectratyping.

Two of the cell lines analyzed, 53 and 68, were derived from the bulk population of 7W-induced CTL that was described above and shown to exhibit differential staining with the parental and 7W tetramers (Fig. 6). Although tetramer analysis of the bulk population suggested that the vast majority of CTL were 7W specific, one of the several T cell clones isolated was cross-reactive. The 53 cell line, which was specific for the 7W variant, was found to express Vβ9. The 68 cell line, which recognized the variant as well as the parental peptide, was found to express Vβ13.6 with completely different CDR3 and J regions from those expressed by line 53 (Table III). Consistent with these findings was the T3

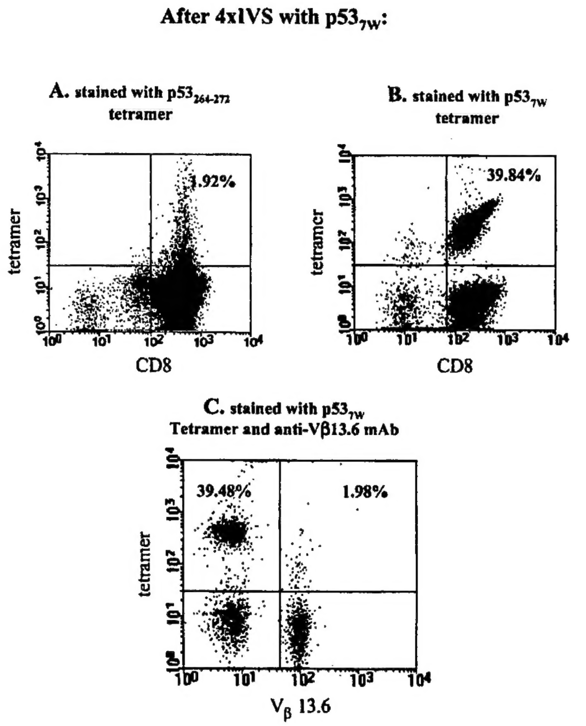

FIGURE 6. CD8$^+$ cells induced from PBMC of a nonresponsive normal donor using the 7W variant peptide that recognizes the parental peptide express TCR Vβ13.6. Three-color flow cytometry analysis of CD8$^+$ cells stained with HLA-A2.1 tetramers containing either the parental peptide (*A*), 7W variant peptide (*B*), or the 7W tetramer and anti-Vβ13.6 mAb (*C*). The numbers in the *upper right quadrants* indicate the percentage of tetramer$^+$ cells. The analyses shown in *A* and *B* involved 75,000 events, while in *C* 10,000 events were analyzed.

result of a combined tetramer/Vβ expression flow analysis of the bulk population from which the 53 and 68 cell lines were derived. We observed that the ~40% cells that stained as a distinct cluster with the 7W tetramer were Vβ13.6$^-$, whereas the ~2% cells that stained weakly with this tetramer were Vβ13.6$^+$ (Fig. 6*C*).

The other two cell lines analyzed, 2 and 4, were derived from bulk populations of effectors induced with the parental peptide from PBMC obtained from a patient (patient 2 in Ref. 12) and a normal donor (donor 2, Table I), respectively. The 2 cell line was found to express Vβ13.6, with identical motifs for the CDR3 and J regions as the 68 cell line, which was induced from a different individual using the 7W peptide (Table III). The observation that cross-reactive T cells induced by the variant peptide from PBMC obtained from one donor express the identical TCR/CDR3/J region as that expressed by T cells induced with the parental peptide from another donor illustrates the heteroclitic nature of the 7W variant peptide.

The 4 cell line was shown to express Vβ1 with a CDR3 and J region sequence distinct from those of any of the other cell lines analyzed (Table III). Interestingly, Vβ analysis of the bulk population of effectors from which the 4 cell line was isolated detected mainly Vβ1 and Vβ13 CD8$^+$ cells. Another bulk population of parental peptide-induced CTL, which was obtained from a different normal donor (7) and used to identify 7W and 6T peptides as potential APLs (Table II), was found to consist of >90% Vβ1$^+$ cells (data not shown). These results, summarized in Table IV, are strongly suggestive of a relatively limited TCR Vβ usage being involved in recognition by CTL of the HLA-A2.1-restricted, wt p53$_{264-272}$ epitope, regardless of whether these cells are induced by the parental or variant peptide.

T4

Discussion

Most studies of APL of tumor Ags involve amino acid exchanges at anchor residue positions of the peptide to enhance its binding to class I MHC molecules. The recent modification of the HLA-A2.1-restricted wt p53$_{149-157}$ peptide at anchor position 2 to improve its binding to the restriction element and immunogenicity is one example of this approach (26). Particularly relevant to this study are variants designed to enhance TCR/peptide interactions rather than increase MHC binding, such as the HLA-A2.1-restricted, melanoma-associated MART1/Melan A$_{27-35}$ and carcinoembryonic Ag, CAP1, peptides, which involve amino acid exchanges in residues other than anchor positions (18–21). While the binding affinities of these variant and parental peptides to HLA-A2.1 molecules are comparable, amino acid exchanges of these peptides at nonanchor positions yielded variant peptides that were more immunogenic than the parental peptides.

Since the parental wt p53$_{264-272}$ peptide has a reasonable affinity for HLA-A2.1 molecules (>1 × 10^{-9} M), the 19 p53$_{264-272}$ variants designed for this study had unmodified anchor positions. Among the amino acid exchanges tested, those at position 6 (6T) and position 7 (7W) appeared to be promising. Since both variants have lower affinities than the parental peptide for HLA-A2.1 molecules, their ability to increase the frequency of anti-p53$_{264-272}$ CTL responses generated from nonresponsive PBMC does not appear to be due to their enhanced binding to HLA-A2.1 molecules. Instead, their increased immunogenicity might be due to the replacement or counterbalancing of residues causing adverse TCR-peptide interactions. Such a replacement could result in an improved interaction of the peptide/MHC complex with TCR and a subsequent expansion of T cells capable of recognizing the parental epitope (23, 24). Two lines of evidence support this conclusion. First, using the parental tetramer to determine the frequency of tetramer$^+$ precursor T cells in unstimulated PBMC obtained from normal donors and patients with cancer, we found that most of the nonresponsive individuals had markedly lower frequencies of these cells in their peripheral circulation than did the responders (data not shown).[4] AQ: F
Second, the parental and variant peptides were found to engage and expand T cells expressing the same TCR in PBMC obtained from responsive and nonresponsive donors (see Tables III and IV). These findings support the concept that increased stability of interaction with the TCR is the basis for the enhanced functional activity of the 7W variant peptide.

Although the use of variant peptides did reverse the nonresponsiveness in IVS of PBMC obtained from some donors, their use did not yield high-affinity CTL. The persistence of low-affinity CTL against self tumor peptides, such as wt p53 epitopes, which is considered a true consequence of tolerance (15), might be due to a limited TCR repertoire being available for recognition of these epitopes. Our analyses detected the predominant use of only two TCR Vβ families, Vβ1 and Vβ13.6, being involved in CTL recognition of the wt p53$_{264-272}$ epitope in four different donors. Furthermore, in two different donors, identical usage by the parental and variant peptide was detected. In contrast, an analysis of responses in HLA-A2.1$^+$ patients to repeated immunizations with an anchor position-variant peptide of the melanoma-associated

[4] T. K. Hoffmann, A. Donnenberg, S. Finkelstein, K. Chikamatsu, V. Donnenberg, U. Friebe, E. Appella, A. B. DeLeo, and T. L. Whiteside. Frequencies of tetramer$^+$ T cells specific for the wild type sequence p53$_{264-272}$ peptide in the circulation of patients with head and neck cancer. *Submitted for publication.*

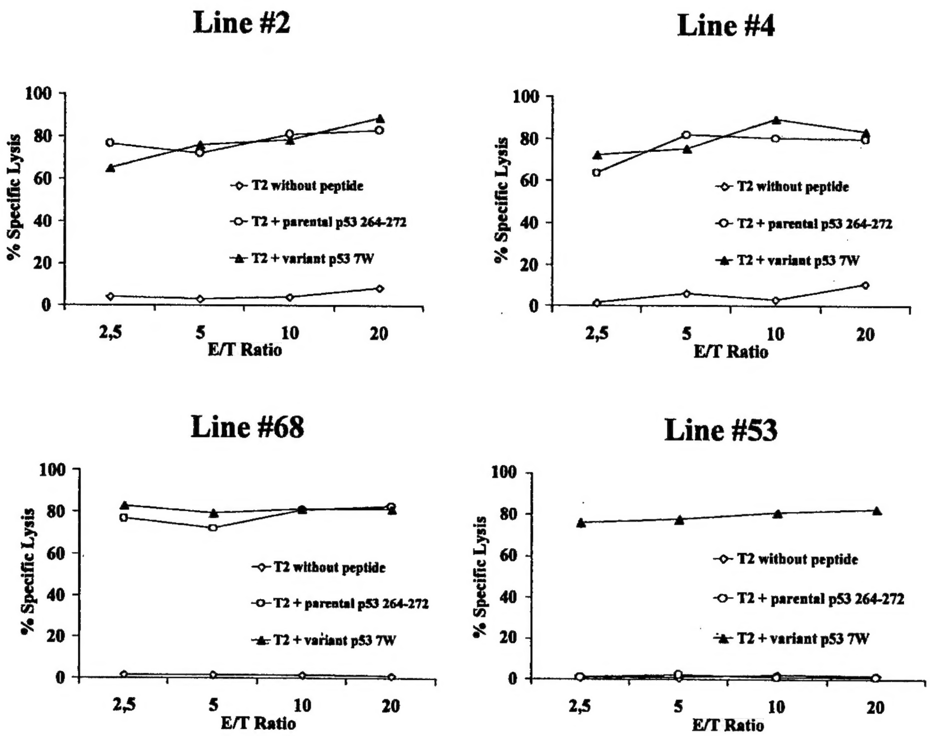

FIGURE 7. Specificity of T cell lines analyzed for TCR Vβ usage. Lines 2 and 4 were derived from bulk populations of effectors induced from an OSCC patient and a normal control, respectively, using the parental peptide. Both are cross-reactive against the variant peptide. Lines 53 and 68 were derived from a bulk population of effectors induced from a nonresponsive healthy donor with the 7W variant peptide. The 53 cell line is specific for the 7W variant, while the 68 cell line is cross-reactive.

$gp100_{209-217}$ epitope demonstrated that the appearance of higher-affinity T cells was associated with an expansion of the TCR repertoire rather than an increased oligoclonal response (33). In the future, additional data on TCR usage of cross-reactive and variant-specific CTL cell could allow for extensive molecular modeling of the interactions within the trimeric complexes and, perhaps, the design of APL with more enhancing properties than those of the 6T and 7W variants. These variants might engage more diverse populations of T cells that are capable of cross-recognition of the parental epitope with, perhaps, higher avidity. However, the apparent outgrowth of epitope-loss tumors in OSCC patients responsive to this epitope suggests that even intermediate-affinity CTL recognizing wt $p53_{264-272}$ might be effective in tumor eradication (12).

To fully estimate the potential of p53-based vaccines in immunotherapy of cancer, it is becoming increasingly apparent that an array of T cell-defined wt p53 epitopes needs to be analyzed, and strategies for optimal induction of T cells recognizing these epitopes need to be further evaluated. In this regard, the use of genetically modified DC expressing intact wt p53 appears to enhance the generation and increase the frequency of antitumor effectors from PBMC of normal donors and cancer patients (34). The p53-based immunotherapy also might be critically dependent on targeting the right epitopes and matching a patient's ability to respond ex vivo to wt p53 epitopes with the potential of his/her tumor to present these epitopes for immune recognition. Again, of course, it is necessary to be aware that a patient's ex vivo responsiveness to these epitopes does not guarantee a successful in vivo response to immunization with them. In this study, HLA-A2.1^{+} patient 3 with OSCC, for whom the tumor cell line and tumor-specific CTL are available in the laboratory, has been of particular interest. The tumor cell line established from this

Table III. *Amino acid sequences of monoclonal TCR transcripts expressed in four parental and/or variant $p53_{264-272}$-specific CTL lines*[a]

				Sequences		
Line	IVS	Specificity	Vβ Family	Vβ	CDR3 region	J region
2	wt	wt + 7W	Vβ13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3
4	wt	wt + 7W	Vβ1	LELGDSALYFCA	SSEGGL	ETQYFGPGTRL/BJ2-5
53	7W	7W	Vβ9	LGDSAVYFCA	SSAGTNT	YEQYFGPGTRLTVT/BJ2-7
68	7W	wt + 7W	Vβ13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3

[a] Lines 2 and 4 were stimulated with the parental wt $p53_{264-272}$ peptide (wt) and were reactive against the parental and 7W variant peptides. Lines 53 and 68 were stimulated with the 7W variant peptide. Line 53 was reactive against the 7W variant peptide only, while line 68 was reactive against the parental and 7W variant peptides. Single peaks in individual TCR variable chain families, suggesting clonality, were analyzed by direct sequencing of the PCR products.

Table IV. *Summary of evidence of limited TCR Vβ usage for CTL recognition of the wt $p53_{264-272}$ epitope*[a]

PBMC Donors[b]	Induced with $p53_{264-272}$ Peptide	Bulk T Cell Population Vβ Usage	Derived T Cell Lines[c] Vβ usage	p53 peptide specificity
Normal donor 2 (R)	wt	Vβ1, 13.6	4 Vβ 1	wt/7W
Normal donor 4[d] (R)	wt	Vβ1	ND	
Normal donor 7 (NR)	7W	Vβ 9, 13.6	53 Vβ 9	7W only
			68 Vβ 13.6[e]	wt/7W
OSCC patient 2[f] (R)	wt	Vβ 13.6	2 Vβ 13.6[e]	wt/7W

AQ: t1

[a] See Table III for the details on TCR Vβ usage.
[b] Normal donors and patients identified in Tables I and II; R, responsive to IVS of PBMC to the wt $p53_{264-272}$ peptide; NR, nonresponsiveness.
[c] T cell lines derived by limiting dilution.
[d] Normal donor used as source of PBMC for induction of a bulk population of CTL specific for wt $p53_{264-272}$. Generation and characterization of this cell line was detailed in Ref. 7.
[e] These T cell lines express identical Vβ, CDR3, and J region sequences (see Table III).
[f] The OSCC patient 2 identified in Table I in Ref. 12.

patient and designated PCI-13 accumulates p53 molecules expressing a missense mutation at codon 286 and naturally presents the $p53_{264-272}$ epitope, albeit following pretreatment with IFN-γ (7, 12). The ability to generate anti-$p53_{264-272}$ CTL with the 7W variant from this patient's PBMC, which were nonresponsive to the parental peptide, provides a basis for the potential use of the 7W variant peptide in immunotherapy of this patient and, perhaps, other nonresponsive OSCC patients with tumors expressing similar characteristics. Concurrently, it needs to be determined whether the trends observed in OSCC patients regarding their responsiveness to wt p53 epitopes and the potential of their tumors to present these epitopes are also apparent in patients with other types of cancers.

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